Neoplasma 2023; 70(6): 733–746

doi:10.4149/neo_2023_230104N8

FBXW7 inhibits the progression of ESCC by directly inhibiting the stemness of tumor cells

Yanghui BI[1,#], Yanfang YANG[2,#], Yong ZHANG[3,#], Caixia CHENG[4], Peisen TANG[5], Heng XIAO[6], Fajia YUAN[7], Weiwei WU[2], Bin YANG[8,*]

[1]General Surgery Department of Third Hospital of Shanxi Medical University, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Taiyuan, Shanxi, China; [2]Department of Epidemiology, School of Public Health, Shanxi Medical University, Taiyuan, Shanxi, China; [3]Endoscopic Center of Shanxi Province Cancer Hospital, Shanxi Hospital Affiliated to Cancer Hospital, Chinese Academy of Medical Sciences, Cancer Hospital Affiliated to Shanxi Medical University, Taiyuan, Shanxi, China; [4]Department of Pathology, The First Hospital, Shanxi Medical University, Taiyuan, Shanxi, China; [5]Department of Biomedical Engineering, City University of Hong Kong, Hong Kong, China; [6]Department of Pathology and Shanxi Key Laboratory of Carcinogenesis and Translational Research of Esophageal Cancer, Shanxi Medical University, Taiyuan, Shanxi, China; [7]Shanxi Province Jinzhong City Health School, Jinzhong, Shanxi, China; [8]3rd Department of Thoracic Surgery Shanxi Province Cancer Hospital, Shanxi Hospital Affiliated to Cancer Hospital, Chinese Academy of Medical Sciences, Cancer Hospital Affiliated to Shanxi Medical University, Taiyuan, Shanxi, China

**Correspondence: yangb8590@163.com*
[#]Contributed equally to this work.

Received January 4, 2023 / Accepted November 13, 2023

F-box and WD repeat domain containing 7 (FBXW7) is an aboriginal and high-frequency mutant gene associated with esophageal squamous cell carcinoma (ESCC). This study was designed to determine the clinical value and molecular mechanisms of FBXW7 in the development of ESCC. The clinical significance of FBXW7 was analyzed in ESCC from TCGA data. The effects of FBXW7 on proliferation, colony formation, migration and invasion, angiogenesis, and apoptosis were tested in ESCC cells. PCR-array, sphere formation assay, and quantitative real-time polymerase chain reaction (qPCR) were used to explore the mechanism of FBXW7. FBXW7 was a significantly mutated gene in ESCC. It was an independent and potential predictor for survival in ESCC patients. In addition, FBXW7 overexpression significantly inhibited ESCC cell proliferation, migration, invasion, angiogenesis, and promoted cell apoptosis. PCR array revealed that FBXW7 overexpression leads to a significant change of gene expressions associated with angiogenesis, cell senescence, and DNA damage and repair. Sphere formation assay and qPCR showed FBXW7 was associated with ESCC stem cell formation. Our results suggest that FBXW7 may act as a tumor suppressor by repressing cancer stem cell formation and regulating tumor angiogenesis, cell senescence, DNA damage, and repair in ESCC.

Key words: FBXW7; ESCC; angiogenesis; stemness

Esophageal cancer is a high-mortality disease that threatens human health. Its morbidity and mortality rank eighth and sixth among all malignant tumors, respectively [1]. Esophageal squamous cell carcinoma (ESCC) is the main pathological subtype of esophageal cancer in China, it is predominant in Fujian and Guangdong, Xinjiang, and Shanxi [2]. At present, the treatment strategy for patients with esophageal cancer is surgery combined with radiotherapy and chemotherapy. However, for patients with tumor progression and metastasis, the treatment effect is poor, and the 5-year survival rate ranges from 15% to 25% [3, 4]. Therefore, the investigation of novel biomarkers for the diagnosis and treatment of ESCC is essential to improve the clinical status of this disease [5].

We previously participated in the whole genome sequencing (WGS) and whole exome sequencing (WES) analyses of 104 ESCC cases. In that study, F-box and WD repeat domain containing 7 (FBXW7*)* was identified as a significantly mutated gene (SMG) associated with ESCC [6]. Moreover, *FBXW7* has been identified as one of the SMGs in other ESCC genomic studies [7–10]. It was suggested that *FBXW7* is a driver gene in ESCC development. *FBXW7*, a substrate component of the SKP1-CUL1-F-box-(SCF)-type ubiquitin ligase (E3) complex that plays a key role in cell division, growth, and differentiation by targeting specific proteins, including c-Myc, Notch1, Notch4, c-Jun, and cyclin E, for degradation of ubiquitination degradation [11]. Recent studies have reported that *FBXW7* acts as a tumor

suppressor gene in various malignant tumors, such as gastric cancer, colon cancer, ovarian cancer, and breast cancer [12, 13]. In gastric cancer, *FBXW7* promoted gastric cancer cell apoptosis, growth arrest and inhibited EMT by inducing RhoA ubiquitination and proteasome degradation [12]. In colon cancer, FBXW7 functioned as a tumor suppressor gene by negatively regulating ENO1 activity via the ubiquitin/proteasome pathway in a GSK3β-dependent manner [14]. In ovarian cancer, *FBXW7* inhibited tumor growth and progression by degrading YTHDF2 to enhance the stability of the pro-apoptotic gene BMF [15]. *FBXW7* inhibits breast cancer proliferation and promotes apoptosis by targeting MTDH for degradation [16]. miR-27a-3p promotes ESCC cell proliferation by inhibiting *FBXW7* [17]. However, the mechanism and clinical significance of *FBXW7* in ESCC remain unclear. Therefore, in this study, we explored the clinical significance of *FBXW7* in ESCC and clarified its function and mechanism, which was expected to provide reliable molecular markers and therapeutic targets for early diagnosis and precise treatment of ESCC.

Materials and methods

Cell lines and culture conditions. All esophageal squamous cell carcinoma cell lines in this study: KYSE150, KYSE180, KYSE450, TE1, TE5, TE6, TE9, TE14, and normal esophageal squamous epithelial cell HET-1A were preserved in the Translational Medicine Center, Shanxi Medical University (Taiyuan, China). All esophageal squamous cell carcinoma cell lines were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, Sigma-Aldrich), and normal esophageal squamous epithelial cell lines were cultured in DMEM high glucose medium (Hyclone, Logan, UT, USA) containing 10% FBS at 37 °C in a 5% CO_2 incubator.

Plasmid transfection. KYSE450 and TE5 esophageal cancer cell lines were seeded in 6-well plates, and when the cell confluence reached 70%, 3 μg PCDNA3-*FBXW7*wt plasmid (950 ng/μl) was used for transfection. The PCDNA3-*FBXW7*wt was purchased from GeneChem company. Plasmids transfection was performed *via* the Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer.

Real-time quantitative PCR (qPCR). The mRNA expression level of *FBXW7* in ESCC cell lines was detected by qPCR. Total RNA was extracted from cells using RNA extraction reagent (RNAiso Plus, Takara, Bio Inc, Japan). qPCR was performed as instructed using the Takara SYBR Premix Ex TaqTM (Takara Bio Inc, Japan) kit for qPCR. All qPCR reactions were replicated using Applied Biosystems (ABI, Foster City, CA, USA). GAPDH served as an internal reference, and the results were calculated using $^{\Delta\Delta}$Ct. Each experiment was repeated at least three times. The primer sequences used in this study are shown in Supplementary Table S1.

Vascular mimicry. 50 μl undiluted Matrigel (BD Biosciences, Bedford, Massachusetts, USA) was added into a 96-well plate and incubated at 37 °C for 30 min. During this period, 1 × 10^4 cells/ml single cell suspension (including 200 μl FBS, 4 μl 1× hydrocortisone, 40 μl 1× hFGF-B, 10 μl 1× VEGF, 10 μl 1× R3-IGF-1, 10 μl 1× ascorbic acid, 10 μl 1× hEGF, 10 μl 1× GA-1000, 10 μl 1× heparin in 10 ml DMEM medium (Lonza, Walkersville, USA)) was prepared. And 50 μl cell suspension was added to 96-well plates with matrix and incubated at 37 °C for 12 h. The tube formation was observed under the microscope, and the images were photographed and recorded, and the number of the tubes was measured by ImageJ software. Each experiment consisted of three replications and was repeated at least three times.

MTT assay. The cells were seeded into 96-well plates at a density of 5×10^3/well and cultured in 5% CO_2 at 37 °C. 20 μl 5 mg/ml MTT (Invitrogen) was added at 24 h, 48 h, 72 h, and 96 h, and incubated at 37 °C for 4 h. The cell culture medium was gently discarded and 150 μl of dimethyl sulfoxide (DMSO) was added to dissolve the crystals. The absorbance at 490 nm was detected by a microplate reader, and the growth curve was drawn according to the OD value to observe the proliferation of cells in each group. Each group contained five multiple wells, and the experiment was repeated at least three times.

Cell migration and invasion assays. Cell migration and invasion abilities were detected by Transwell assay. In the migration assay, 5×10^4 cells were seeded in a basic medium without FBS in a 24-well plate chamber, and 600 μl of medium with 10% FBS was added to the bottom chamber. 3 replicate wells were set up for each group. Cells were incubated in 5% CO_2 at 37 °C for 24 h. 4% paraformaldehyde was added to the upper chamber to fix the cells, and 0.1% crystal violet solution was used for staining. When the samples were observed under a microscope (Olympus, Japan), 8 areas were randomly selected to count the cell number. In the invasion assay, the chambers were pre-coated with 50 μl Matrigel (1:6 mixed with serum-free medium, BD Biosciences, Heidelberg, Germany) and performed as described above. Each group included 3 repeated wells, and the experiment was repeated 3 times independently.

Colony formation assay. Cells were seeded in 6-well plates at a density of 1,000 cells/well and cultured in 5% CO_2 at 37 °C for 2 weeks. The cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. The number of effective clones formed by cells in each group was observed and counted under a microscope, and the clonal ability of cells in each group was compared. Each experiment consisted of 3 replicates with at least 3 experiments.

Flow cytometry apoptosis detection. ESCC cells were detached with EDTA-free trypsin, centrifuged to collect cell pellet, washed with PBS, resuspended with 500 μl binding buffer, mixed with 5 μl of Annexin V-FITC (Solarbio life Science, China), and 10 μl of Propidium Iodide (Solarbio Life Science, China). After incubation in the dark at room temperature for 20 min, the cells were detected by flow

cytometry (Beckman Coulter Life Science, USA). We did this experiment at least three times.

Tumorsphere formation. The logarithmic phase cancer cells were seeded into a 6-well ultra-low attachment plate (Corning) and cultured with DMEM-F12 medium (Gibco, USA) containing 20 ng/ml human epidermal growth factor (hEGF) (PEPROTECH), 20 ng/ml basic fibroblast growth factor (bFGF) (PEPROTECH), 1×B27 and 5 μg/ml insulin (Gibco) at a density of 5×10^3 ml^{-1}. 1 ml fresh culture medium was added to the culture plate every 3 days. For 7 to 14 days, cells were grown in suspension. The diameters of the spheres were measured using an inverted microscope. A sphere with a diameter greater than 50 μm was counted. We did this experiment at least three times.

PCR array. To screen for differentially expressed genes associated with *FBXW7*, we performed a PCR array using the RT² Profiler™ PCR Array Human Cancer Pathway Finder Kit (PAHS-033ZC) provided by Shanghai QIAGEN Biotechnology Company. According to the operation instructions: Total RNA was extracted by TRIzol, and then reverse transcription into cDNA with PrimeScript RTMasterMix (Takara, Shiga, Japan). The cDNA template was diluted and added to the real-time quantitative PCR reaction mixture, and then 25 μl reaction solution was added to each well of the RT² Profiler PCR Array containing gene-specific primers for real-time quantitative PCR reaction. The results were analyzed using online analysis software (https://geneglobe.qiagen.com/cn/).

Statistical analysis. Expression data of *FBXW7* gene in ESCC and the corresponding clinicopathological data were collected from TCGA via xenabrowser (https://xenabrowser.net/heatmap/) [18]. The protein-protein interaction network was analyzed based on Differently Expressed Genes (DEGs). SPSS 26.0 software was used for statistical analysis. The experiment was divided into three replicates and the data were expressed as mean ± SEM. Rank sum test, Breslow test, and chi-square test (χ^2) were used to analyze the relationship between *FBXW7* expression and clinical pathological characteristics. Non-paired t-test was used for the data of the two groups, and one-way ANOVA was used for the data above the two groups. A p-value <0.05 was considered statistically significant.

Results

Low *FBXW7* expression reflects poor prognosis in patients with ESCC. In a previous study in which we were particular, WGS/WES analysis was performed in 104 ESCC cases, and 8 SMGs were identified (false discovery rate <0.178, p<0.0001). The mutation frequency of *FBXW7* was 7.7% (8/104), covering nonsense mutations [2/8 (25%)], insertion/deletion [1/8 (12.5%)], and missense mutations [5/8 (62.5%)] (Figure 1A) [19, 20]. In addition, the RNA-sequencing (RNA-seq) results from 154 ESCC cases indicated that the *FBXW7* mRNA level in tumor tissues was significantly lower than those noted in adjacent normal tissues (Figure 1B).

Table 1. Correlation analysis between FBXW7 mRNA levels in ESCC and clinicopathological variables.

Characteristic	Total (n=95)	FBXW7 expression (n%)		χ^2	p-value
		Low (≤324.7751) n=23	High (>324.7755) n=72		
Age					
<55	40	11 (27.50)	29 (72.50)	0.41	0.52
≥55	55	12 (21.82)	43 (78.18)		
Gender					
male	81	21 (25.93)	60 (74.07)	0.36	0.55
female	14	2 (14.29)	12 (85.71)		
Smoking					
no	11	6 (54.55)	5 (45.46)	0.45	0.80
yes	84	57 (67.86)	27 (32.14)		
Drinking					
no	69	16 (23.19)	53 (76.81)	0.34	0.56
yes	26	9 (34.62)	17 (65.39)		
TNM Stage					
I+II	62	16 (25.81)	46 (74.19)	0.12	0.73
III+IV	33	9 (27.27)	24 (72.73)		
T stage					
1+2	39	10 (25.64)	29 (74.36)	0.03	0.86
3	56	15 (26.79)	41 (73.21)		
Prognosis					
survival	63	11 (17.46)	52 (82.54)	4.64	0.03
dead	32	2 (37.50)	20 (62.50)		

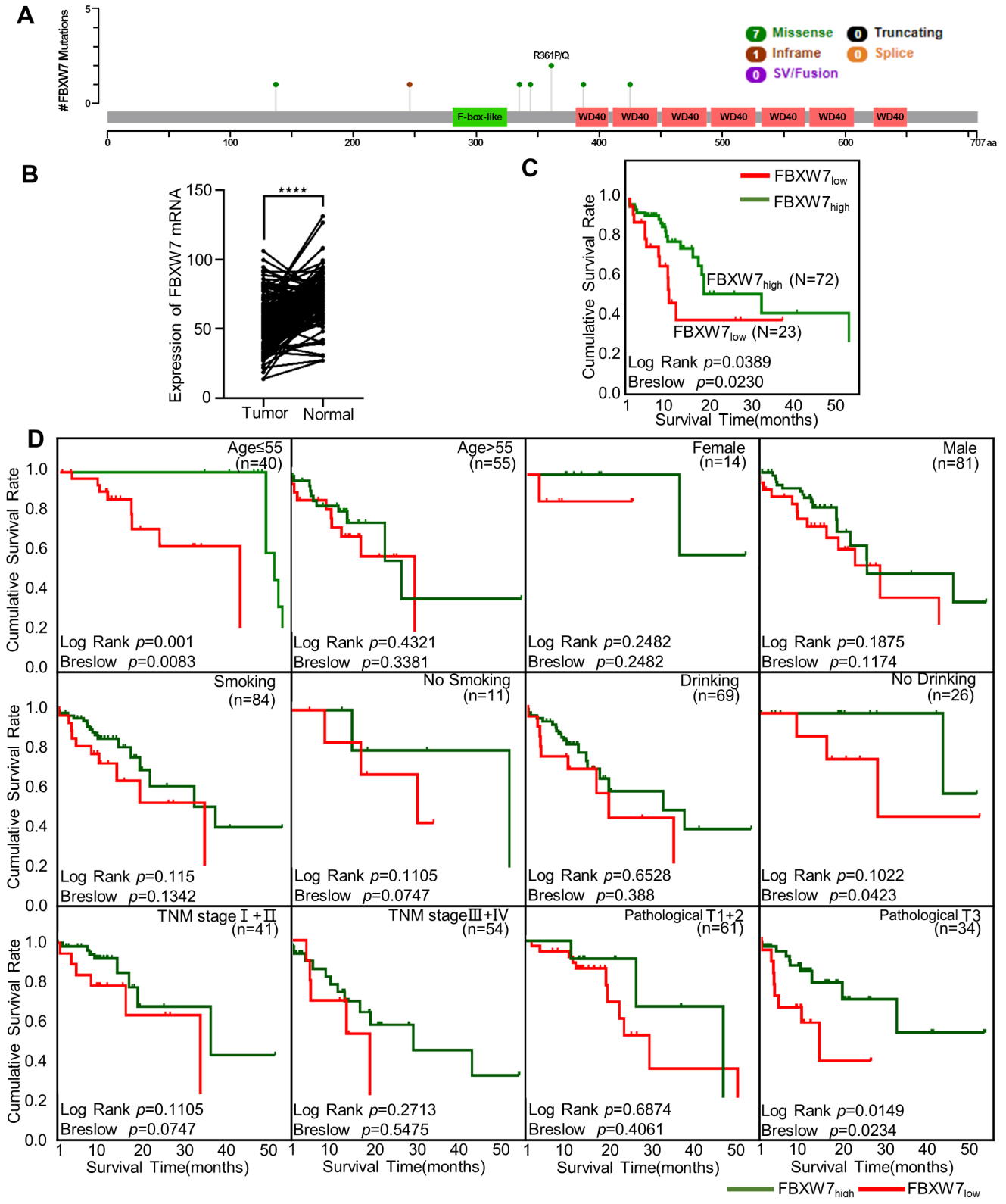

Figure 1. Low expression of FBXW7 was associated with poor prognosis in ESCC patients. A) Mutation site distribution of FBXW7 gene in 104 ESCC samples. B) Analysis of the mRNA expression of FBXW7 in 154 pairs of ESCC tissues. C) FBXW7 expression level and overall Kaplan-Meier survival analysis of ESCC patients. D) Kaplan-Meier survival curves of patients with different FBXW7 expression in different age, gender, smoking history, drinking history, tumor grade, and pathological T stage.

In order to assess, the clinical significance of *FBXW7* in ESCC, rank sum, Breslow test, and Chi-square (χ^2) tests were applied in the ESCC cohort (95 cases) from TCGA database. According to the expression of *FBXW7* and the survival information of patients with ESCC, the ROC curve analysis was performed and the critical value of *FBXW7* expression was determined (cutoff = 324.78). The patients with ESCC were divided into low expression (≤324.78) and high expression (>324.78) groups. Subsequently, the correlation between *FBXW7* expression and ESCC clinical variables was analyzed; the data indicated that *FBXW7* expression correlated with the survival status (p=0.031; Table 1). Survival analysis indicated that patients with low *FBXW7* expression in ESCC exhibited a worse prognosis than those with high *FBXW7* expression (p<0.05; Figure 1C). In addition, further stratification analysis indicated that the prognosis of patients with ESCC with low *FBXW7* expression was poor in patients ≤55 years old, without drinking history and pathological T3 (p<0.05; Figure 1D). However, no significant differences were noted in the variables gender, smoking history, and TNM stage of patients with ESCC between the high *FBXW7* expression and the low *FBXW7* expression groups.

These results suggested that the low expression of *FBXW7* may be an independent prognostic factor for the poor prognosis of patients with ESCC.

***FBXW7* inhibited cell proliferation, migration, and invasion and promoted apoptosis in ESCC.** mRNA expression levels of *FBXW7* were examined in 9 ESCC cell lines and the data demonstrated that KYSE450 and TE5 cells had low endogenous *FBXW7* mRNA expression levels (p<0.001; Figure 2A). Therefore, the pcDNA3-*FBXW7* wild-type (wt) was transfected into KYSE450 and TE5 cells to explore the corresponding gene functional effects. The overexpression efficiency was confirmed by qPCR (p<0.05; Figure 2B). It was shown that *FBXW7* overexpression (FBXW7-OE) significantly inhibited the viability and self-renewal of KYSE450 and TE5 cells by MTT and colony formation assays (p<0.05; Figures 2C, 2D). Moreover, the results of the Transwell experiments indicated that *FBXW7* overexpression was able to inhibit the invasive and migratory ability of ESCC cells (p<0.05; Figures 2E, 2F). The induction of apoptosis in cells overexpressing *FBXW7* was detected by Annexin V-FITC/PI double staining using flow cytometry; the data indicated that the number of apoptotic cells was significantly increased (Figure 2G), suggesting that *FBXW7* may be a tumor suppressor gene in ESCC development.

Altered pathways and gene-interaction networks are affected by *FBXW7* overexpression in ESCC cells. On the basis of clarifying the function of *FBXW7*, we performed PCR array experiments with *FBXW7-OE* and control cells to explore the mechanism of *FBXW7* in ESCC. The results indicated the identification of 16 genes with >2-fold changes in expression levels; the genes were as follows: *ANGPT1*, *SERPINB2*, *SOD1*, *DDB2*, *DDIT3*, *ERCC3*, *ERCC5*, *POLB* and *PPP1R15A*, *FLT1*, *KDR*, *PGF*, *TEK*, *BMI1*, *IGFBP5,* and *TBX2*. They were mainly concentrated in angiogenesis, DNA damage repair, and cell senescence-related signaling pathways (Figures 3A, 3B), suggesting that FBXW7 may be related to these pathways in ESCC. This result was also verified by a qPCR experiment (Figure 3C).

In addition, the gene interaction network between *FBXW7* and these genes was investigated using Cytoscape. As shown in Figure 4A, in the angiogenesis network, in addition to the central *FBXW7* gene, there were other core genes were identified, including upregulated *ANGPT1* and downregulated *FLT1*, *KDR*, *PGF*, and *TEK*. In the cell senescence network, core genes also included upregulated *SOD1*, *SERPINB2*, and downregulated *IGFBP5* (Figure 4B). In the DNA damage repair network, the core genes also included upregulated *DDIT3*, *PPP1R15A*, *POLB*, *ERCC5*, and *DDB2* (Figure 4C). Collectively, the aforementioned results indicated that FBXW7 may play a tumor suppressor role via angiogenesis, cell senescence, DNA damage, and repair signaling pathways in ESCC.

***FBXW7* regulated angiogenesis, cell senescence, and DNA damage repair pathways by inhibiting tumor stemness in ESCC.** Subsequent experiments investigated the association of tumor stemness with the effect of FBXW7 on angiogenesis and cell senescence-related pathways in ESCC cells. The vascular mimicry assay was performed to examine the effect of *FBXW7* on angiogenesis in FBXW7-OE and control cells. The results indicated that the number of tubular structures in the FBXW7-OE group was significantly reduced compared with the control group, suggesting that *FBXW7* could inhibit angiogenesis in ESCC (Figure 5A). Furthermore, we cultured KYSE450 and TE5 tumorspheres, and the data indicated that compared with the parental cells, the volume of tumorspheres formed in the KYSE450-FBXW7-OE group was smaller, and the number of tumorspheres formed in the TE5-FBXW7-OE group was less (p<0.05) (Figure 5B). These results suggested that the negative regulation of FBXW7 on cell stemness may be a universal mechanism in ESCC.

Discussion

In the present study, the significant mutation gene *FBXW7* was identified in ESCC by analyzing our previous genomic sequencing data. The findings uncovered its potential prognostic value for patients with ESCC. Specific *in vitro* methods were applied to reveal the tumor suppressor role of *FBXW7* and a possible novel mechanism of regulating ESCC angiogenesis, cell senescence, and DNA damage repair by affecting the stemness of ESCC cells (Figure 6).

FBXW7 also known as Sel10, hCDC4, or hAgo, is a member of the F-box protein family, which functions as the substrate recognition component of the SCF E3 ubiquitin ligase [21]. Previous WGS or WES studies revealed a series of mutated genes of ESCC. *FBXW7* was identified as one of the SMGs present in the cohort examined in our study. Subse-

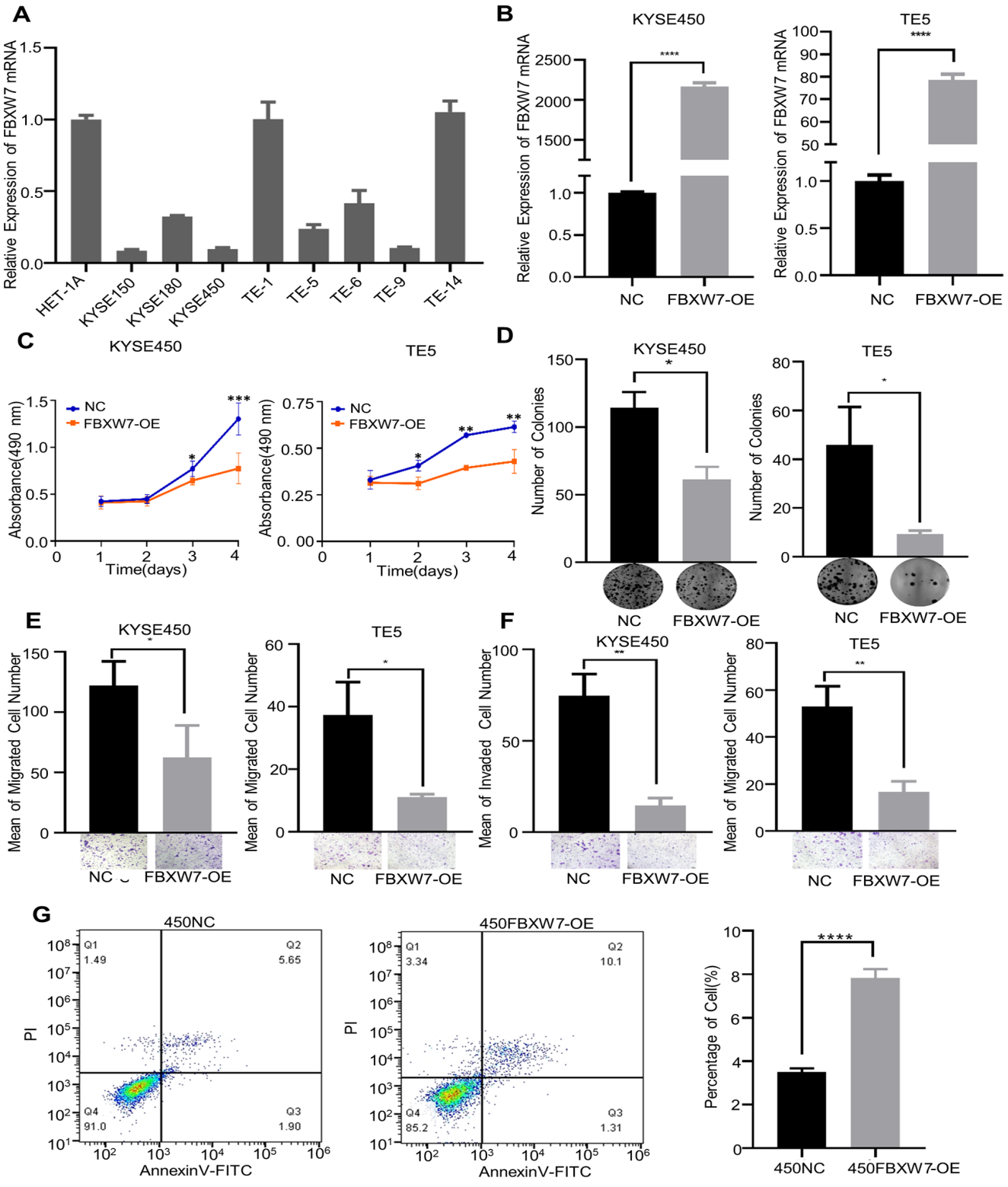

Figure 2. Function of FBXW7 in ESCC. A) FBXW7 mRNA expression levels in 9 ESCC cell lines. B) The overexpressed efficiency of FBXW7 in KYSE450 and TE5 cells was detected by qPCR. C) Overexpression of FBXW7 inhibited the proliferation of ESCC cells. D) Overexpression of FBXW7 inhibited colony formation of ESCC cells. E) Overexpression of FBXW7 inhibited the migration of ESCC cells F) Overexpression of FBXW7 inhibited the invasion of ESCC cells. G) Overexpression of FBXW7 in KYSE450 cell line, the proportion of apoptosis was significantly increased *p<0.05, **p<0.01

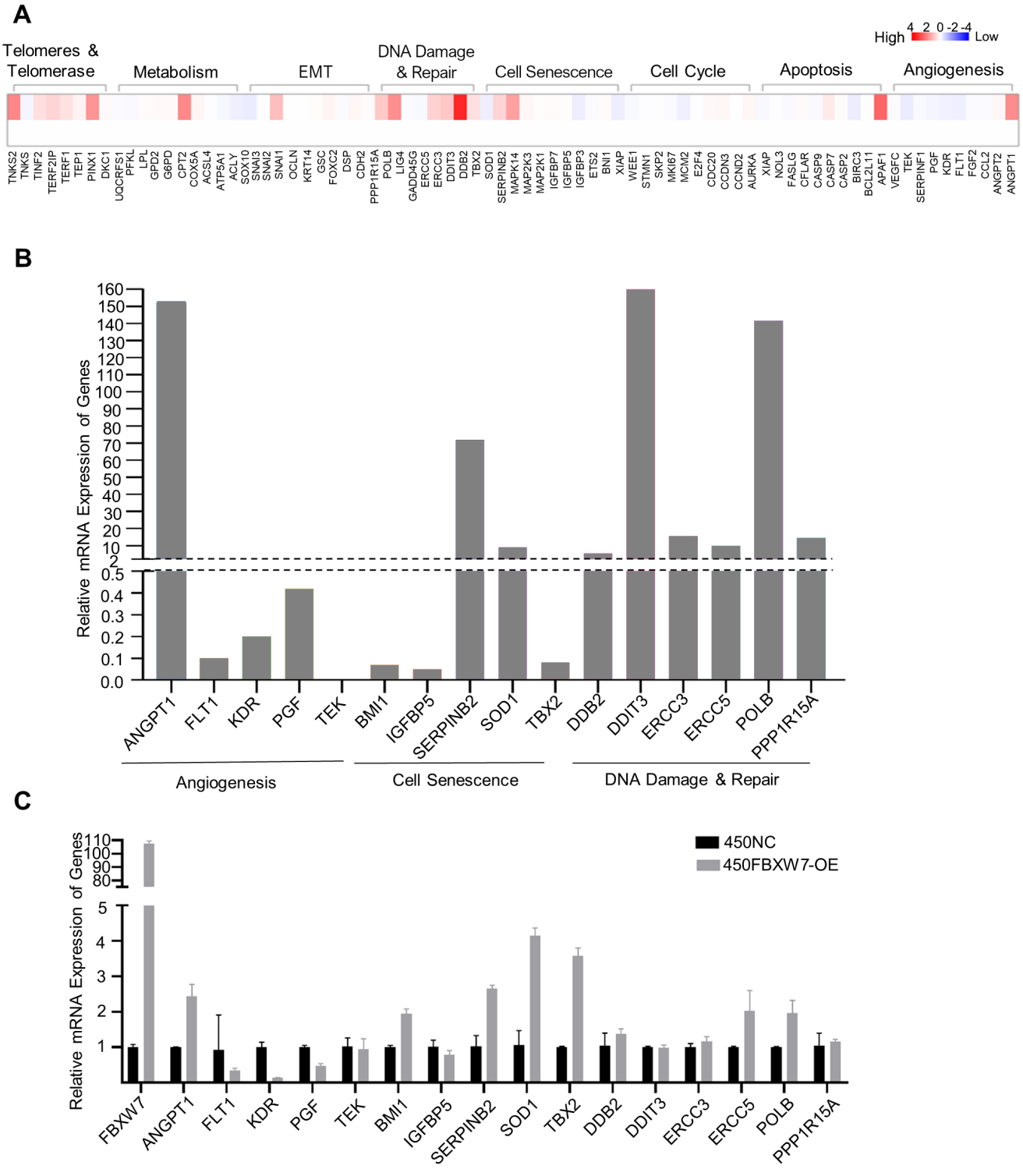

Figure 3. The analysis of key cancer pathway PCR array kit components altered in FBXW7 overexpressing cells. A) PCR arrays were used to detect gene expression affected by FBXW7. Blue-white-red scales represent fold change and fold regulation values for genes. The blue-white-red scale represents the fold-change and the fold regulation value of genes. Blue: Fold downregulation (fold change <1); White: Fold change = 1; Red: Fold upregulation (fold change >1). B) PCR array was used to show the key cancer pathway correlating with FBXW7. C) q-PCR was used to detect the mRNA level of genes in KYSE450NC and KYSE450-FBXW7-OE. GAPDH was used as a loading control. All data are presented as the mean ± standard deviation and three independent experiments. *p<0.05, **p<0.01, ***p<0.001

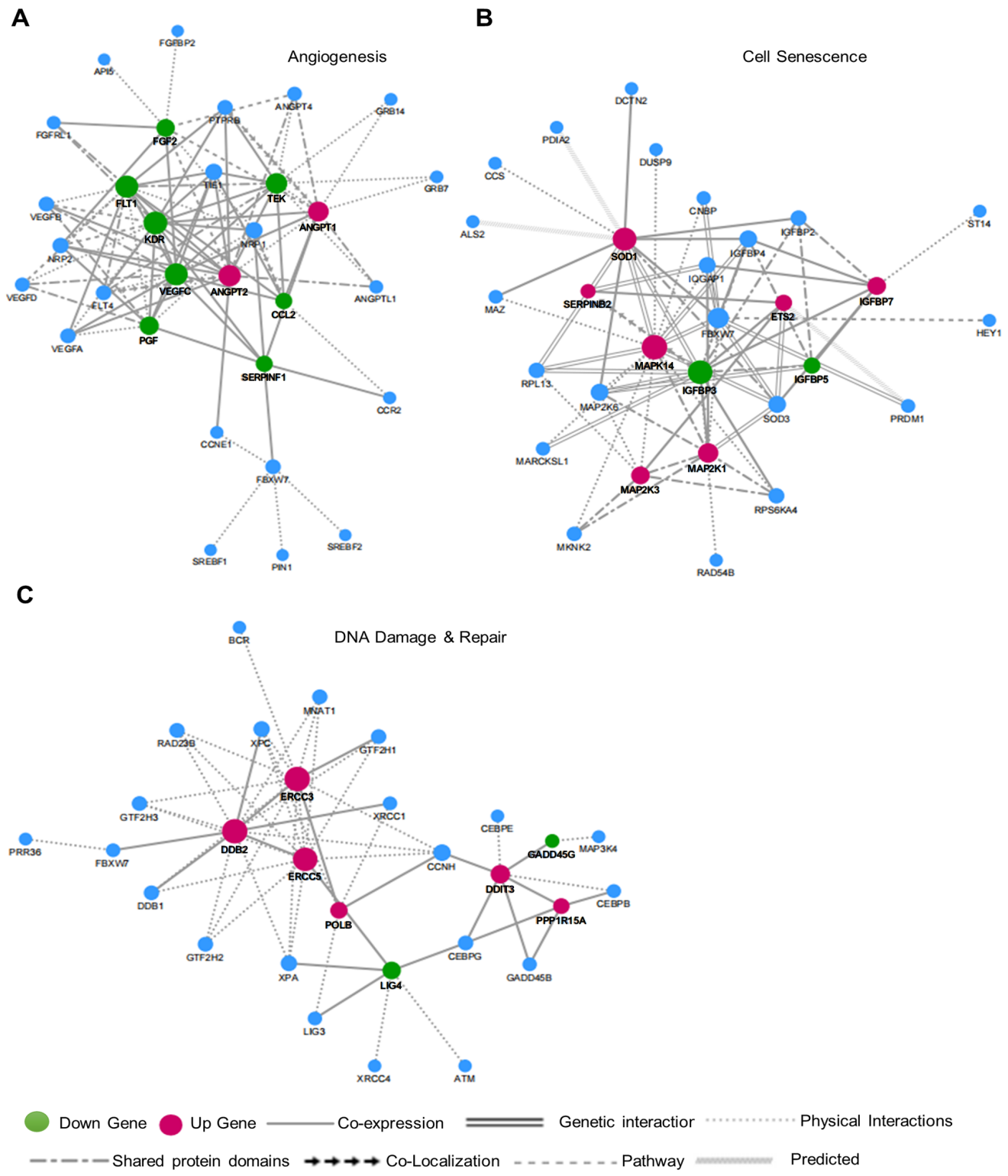

Figure 4. Gene interaction network analysis of FBXW7. A) The network of FBXW7 gene interactors is mainly concentrated in the angiogenic pathway. B) The network of FBXW7 gene interactors is mainly focused on the signaling pathways of cellular senescence. C) The network of FBXW7 gene interactors is mainly concentrated in the DNA damage repair pathway. Upregulated genes are indicated in red, downregulated genes are indicated in green, and blue indicates no significant difference. The size of the circle represents the change in folding.

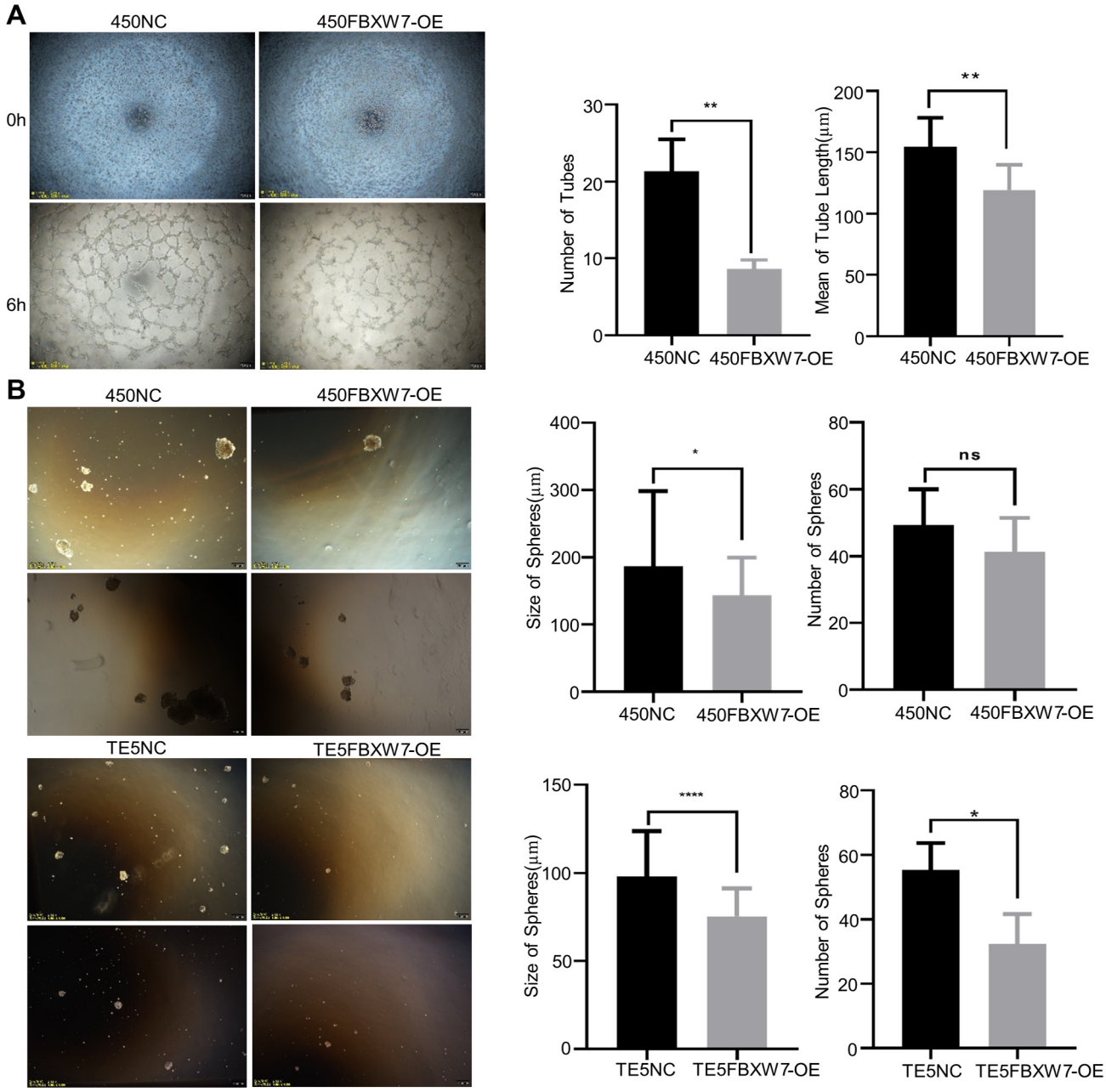

Figure 5. The effect of FBXW7 on ESCC stemness. A) Left panel: Representative images of vasculogenic mimicry experiments. Right panel: Quantitative results of tube formation using ImageJ software. B) Left panel: Representative images of tumorsphere formation experiments. Right panel: Quantitative results of the size of tumorsphere using ImageJ software. *p<0.05, **p<0.01

quently, our expanded cohort study of 508 ESCC samples revealed that the mutation frequency of FBXW7 was 8.66% [6]. The genomic sequencing data from 4 ESCC cohorts was analyzed and it was found that *FBXW7* was mutated frequently in ESCC. In the cohort reported by Du et al. [9], 490 tumor samples and matched non-tumor samples were used and the mutation rate of *FBXW7* was approximately 5.31%. In the cohort reported by Gao et al. [7], 113 tumor samples and matched non-tumor samples were investigated and the mutation rate of *FBXW7* was approximately 5%. In the cohort examined by Agrawal et al. [22], 41 tumor samples and matched non-tumor samples were assessed and the mutation rate of *FBXW7* was approximately 6%. In the cohort examined by Sawada et al. [8], 144 tumor samples and matched non-tumor samples were examined and the mutation rate of *FBXW7* was approximately 5.6%. The RNA-seq results

of 155 pairs of ESCC samples indicated that the expression levels of *FBXW7* in tumor samples were lower than their matched normal samples. Clinically, it was observed that low *FBXW7* expression was associated with poor prognosis in ESCC, and individuals with low *FBXW7* expression exhibited considerably worse prognosis than individuals with high *FBXW7* expression in young, non-drinking, and later-stage populations. Consistent with our analysis, the clinical significance of *FBXW7* expression-related prognosis was evaluated in 75 patients with ESCC and it was shown that the loss of the FBXW7 copy number in 45 patients with ESCC was related to disease prognosis [23]. The 5-year survival rates of the 90 patients with high and low FBXW7 expression were 67.6% and 39.3%, respectively (χ^2=6.699, p=0.01) [24].

It has been reported that *FBXW7* is a suppressor gene in gastric cancer, colon cancer, ovarian cancer, breast cancer, liver cancer, and other malignant cancer types [15, 25, 26]. These findings were consistent with the results of the functional assays, demonstrating results that *FBXW7* also functions as a suppressor gene in ESCC, inhibiting ESCC cell proliferation, invasion and migration, and promoting apoptosis. Accordingly, the PCR-array results and the validation tests indicated that *FBXW7* may contribute to ESCC tumorigenesis via angiogenesis, DNA damage repair, and cell senescence-related signaling pathways. It was deduced that *FBXW7* interacted with sequence-specific transcription factors and suppressed activation of angiogenesis and DNA damage repair, which promotes activation of cell senescence-relevant genes; these processes participated in the tumorigenesis and development of ESCC. The genes with altered expression levels involved in angiogenesis were examined in *FBXW7-OE* ESCC cells. The results revealed the downregulation of *FLT1*, *KDR*, *PGF*, and *TEK*, and the upregulation of *ANGPT1*. *FLT1*, the receptor of vascular endothelial growth factor (VEGF)/phosphatidyl inositol glycan anchor biosynthesis class F (PIGF) is associated with cancer angiogenesis and tumorigenesis [27]. *KDR* is not only expressed in endometrial carcinoma cells but is also actively participating in the biological process of the tumor [28]. Abnormal expression of *PGF* is noted in cervical carcinoma [29]. *TEK* promotes cell apoptosis by regulating AKT phosphorylation, thereby inhibiting cell proliferation [30]. The expression levels of *ANGPT1* are significantly downregulated in patients with lung squamous cell carcinoma and lung adenocarcinoma [31]. These findings were consistent with the trend of the expression changes noted in the genes of our study indicating the inhibitory effect of *FBXW7* on angiogenesis. In the DNA damage repair network, the core genes also included the following genes which were upregulated: *DDIT3*, *PPP1R15A*, *POLB*,

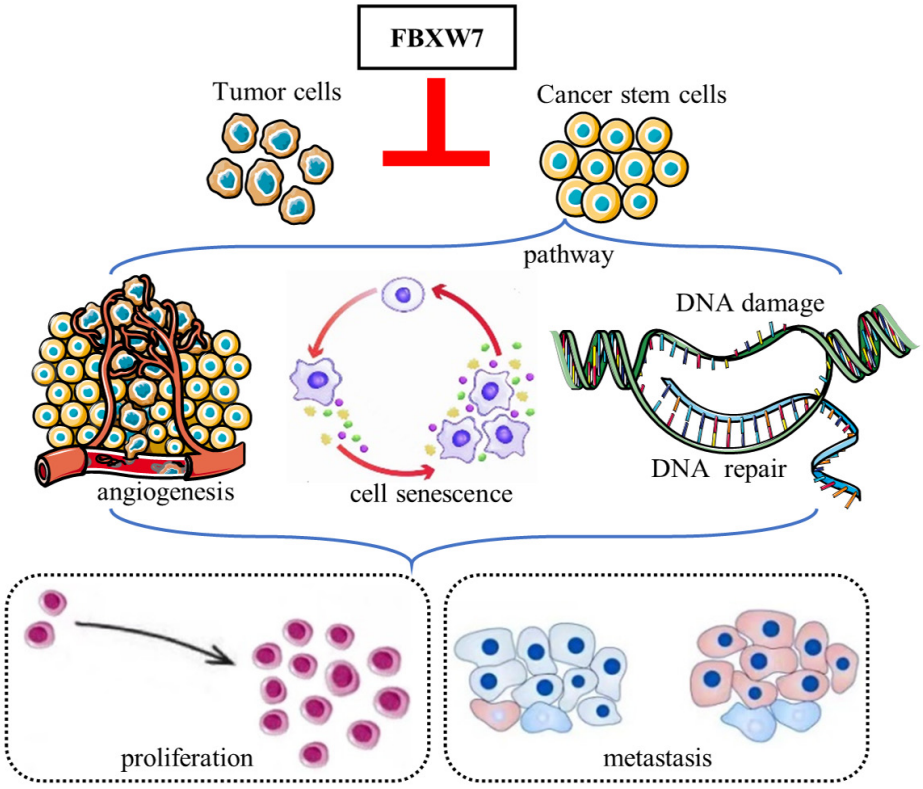

Figure 6. The tumor suppressor role of FBXW7 and a possible novel mechanism of regulating ESCC angiogenesis, cell senescence, and DNA damage repair by affecting the stemness of ESCC cells.

ERCC5, and *DDB2*. Overexpression of *DDIT3* enhanced gastric cancer cell proliferation, colony formation, spheroid formation, and CSC stemness [32]. *PPP1R15*A plays a vital role in promoting cell death and the unfolded protein response (UPR) [33]. *POLB* regulates the growth of oral squamous cell carcinoma cells by regulating cell cycle and chromosome instability [34]. *ERCC5* polymorphism may be associated with metastasis and recurrence of gastric cancer [35]. *DDB2* plays a role as a transcription inhibitor and can eliminate the characteristics of ovarian CSC by downregulating the expression of ALDH1A1 [36]. These reports are consistent with the altered trend of gene expression in the present study, indicating that *FBXW7* can activate DNA damage repair. In the cell senescence network, core genes also included *SOD1* and *SERPINB2* (upregulated), and *IGFBP5* (downregulated). Inhibition of *SOD1* induces cell death by activation of apoptosis [37]. Overexpression of *SERPINB2* reduces survival rate and increases lymph node metastasis in patients with breast cancer patients [38]. *IGFBP5* regulates the radiosensitivity of prostate cancer through the PI3K-AKT pathway [39]. These reports back with the findings of this study, which show that FBXW7 can enhance the activation of cell senescence-related genes.

It is well known that angiogenesis, DNA damage repair, and cell senescence are biological processes that participate in tumorigenesis. The coenzyme Q10 analog decyl ubiquinone (DUb) inhibits angiogenesis by regulating the ROS/p53/BAI1 signaling pathway, thereby inhibiting the occurrence and development of breast cancer [40]. The pathways of DNA damage repair determining cell fate are intertwined and play a key role in the occurrence and development of cancer [41]. In cancer, cell senescence is an effective barrier to prevent the occurrence of tumors and can also drive the development of proliferative diseases [42]. Therefore, we further analyzed the interaction network between FBXW7 and the related pathway genes. The results revealed that FBXW7 may be a considerable factor in angiogenesis, DNA damage, and cell senescence pathway, forming a complex regulatory network and playing an important role in ESCC. Hu et al. demonstrated that FBXW7-OE reduced angiogenesis in the retinal tissues of mice with diabetic retinopathy [43]. Therefore, we applied the vascular mimicry experiments were applied to determine the effect of FBXW7 on the angiogenesis of ESCC cells and the data indicated that FBXW7 could inhibit the angiogenic ability of ESCC cells. In response to DNA damage, phosphorylated FBXW7 can play a role in maintaining genomic stability [44]. Following induction of radiation or oxidative stress, FBXW7 binds to the telomeric protective protein 1 (TPP1) and triggers telomere uncapping to mediate cellular senescence [45].

However, several studies have reported that angiogenesis, DNA damage, and the cell senescence pathway were involved in CSCs formation in various malignant tumors [46]. In gastric cancer, tumor stem cells exhibit greater tumor formation and angiogenesis than cancer cells [47]. Tumor stem cells are responsible for the inhibition of angiogenesis caused by the Notch signaling pathway in breast cancer [48]. Additionally, Mateusz et al. reported VEGF, ANGPT1, ANGPT2, and MMP-9 expression in the autologous hematopoietic stem cell transplantation and its impact on the time to engraftment [49]. With regard to cell senescence, colorectal cancer stem cells can promote apoptosis and cellular senescence by regulating the FasR/FasL signaling pathway. Liu et al. reported that tetrandrine inhibits cancer stem cell characteristics and epithelial-to-mesenchymal transition in triple-negative breast cancer via the SOD1/ROS signaling pathway [50]. Hao et al. declared that IGFBP5 enhances the dentinogenesis potential of dental pulp stem cells via JNK and ErK signaling pathways [51]. Tumor stem cells can also effectively regulate DNA damage repair. Hematopoietic stem cells are affected by the ubiquitination enzyme USP16, which removes ubiquitin from histone 2A (H2A) and H2AX and thereby impairs DNA damage repair [52]. Han et al. reported that DDB2 suppresses tumorigenicity by limiting the cancer stem cell population in ovarian cancer. Lin et al. suggested that DDIT3 modulates cancer stemness in gastric cancer by directly regulating CEBPb [53]. Therefore, the effect of *FBXW7* was investigated on the stemness of ESCC cells. Further studies indicated that overexpression of *FBXW7* could inhibit the formation and proliferation of ESCC tumorspheres.

In summary, the functional and clinical analysis of the current study suggests that *FBXW7* may regulate the occurrence and development of ESCC by inhibiting the stemness of ESCC cells subsequently affecting the signaling pathways related to ESCC angiogenesis, DNA damage repair, and cell aging. Moreover, low expression of *FBXW7* was associated with a poor prognosis. These findings are important for understanding the mechanisms that drive the development and progression of ESCC, providing novel information for unraveling the mechanisms of tumorigenesis and tumor progression and highlighting new applications for the clinical treatment of ESCC.

Supplementary information is available in the online version of the paper.

Acknowledgments: This work was financially supported by the National Foundation of Natural Science of China (82002606 to Yanghui Bi), Shanxi Province "136 Revitalization Medical Project Construction Funds" (2021YZ17 to Yanghui Bi), Science and Technology Innovation Project of Universities in Shanxi (2021RC010 to Yanghui Bi), Shanxi Province Cancer Hospital, China Academy of Medical Sciences Cancer Hospital Shanxi Hospital Master Guide Flying Fund (SD2023007 to Bin Yang). This fund provides various experimental technology platforms as well as various instruments and equipment required for the experiment. The authors would like to thank the Shanxi Provincial Science and Technology Innovation Key Team of Digestive System Malignant Tumor Prevention and Control for the critical reading of the manuscript and suggestions.

References

[1] SUNG H, FERLAY J, SIEGEL RL, LAVERSANNE M, SOERJOMATARAM I et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 2021; 3: 209–249. https://doi.org/10.3322/caac.21660

[2] ZHAO P, DAI M, CHEN W, NI L. Cancer trends in China. Jpn J Clin Oncol 2010; 4: 281–285. https://doi.org/10.1093/jjco/hyp187

[3] GUPTA B, KUMAR N. Worldwide incidence, mortality and time trends for cancer of the oesophagus. Eur J Cancer Prev 2017; 2: 107–118. https://doi.org/10.1097/CEJ.0000000000000249

[4] OHASHI S, MIYAMOTO S, KIKUCHI O, GOTO T, AMANUMA Y et al. Recent Advances From Basic and Clinical Studies of Esophageal Squamous Cell Carcinoma. Gastroenterology 2015; 7: 1700–1715. https://doi.org/10.1053/j.gastro.2015.08.054

[5] JIA J, LI H, CHU J, SHENG J, WANG C et al. LncRNA FAM83A-AS1 promotes ESCC progression by regulating miR-214/CDC25B axis. J Cancer 2021; 4: 1200–1211. https://doi.org/10.7150/jca.54007

[6] CUI Y, CHEN H, XI R, CUI H, ZHAO Y et al. Whole-genome sequencing of 508 patients identifies key molecular features associated with poor prognosis in esophageal squamous cell carcinoma. Cell Res 2022; 4: 415–416. https://doi.org/10.1038/s41422-022-00625-x

[7] GAO YB, CHEN ZL, LI JG, HU XD, SHI XJ et al. Genetic landscape of esophageal squamous cell carcinoma. Nat Genet 2014; 10: 1097–1102. https://doi.org/10.1038/ng.3076

[8] SAWADA G, NIIDA A, UCHI R, HIRATA H, SHIMAMURA T et al. Genomic Landscape of Esophageal Squamous Cell Carcinoma in a Japanese Population. Gastroenterology 2016; 5: 1171–1182. https://doi.org/10.1053/j.gastro.2016.01.035

[9] DU P, HUANG P, HUANG X, LI X, FENG Z et al. Comprehensive genomic analysis of Oesophageal Squamous Cell Carcinoma reveals clinical relevance. Sci Rep 2017; 1: 15324. https://doi.org/10.1038/s41598-017-14909-5

[10] HU N, KADOTA M, LIU H, ABNET CC, SU H et al. Genomic Landscape of Somatic Alterations in Esophageal Squamous Cell Carcinoma and Gastric Cancer. Cancer Res 2016; 7: 1714–1723. https://doi.org/10.1158/0008-5472.CAN-15-0338

[11] FAN J, BELLON M, JU M, ZHAO L, WEI M et al. Clinical significance of FBXW7 loss of function in human cancers. Mol Cancer 2022; 1: 87. https://doi.org/10.1186/s12943-022-01548-2

[12] LI H, WANG Z, ZHANG W, QIAN K, XU W et al. Fbxw7 regulates tumor apoptosis, growth arrest and the epithelial-to-mesenchymal transition in part through the RhoA signaling pathway in gastric cancer. Cancer Lett 2016; 1: 39–55. https://doi.org/10.1016/j.canlet.2015.10.006

[13] CAO J, GE MH, LING ZQ. Fbxw7 Tumor Suppressor: A Vital Regulator Contributes to Human Tumorigenesis. Medicine (Baltimore) 2016; 7: e2496. https://doi.org/10.1097/MD.0000000000002496

[14] ZHAN P, WANG Y, ZHAO S, LIU C, WANG Y et al. FBXW7 negatively regulates ENO1 expression and function in colorectal cancer. Lab Invest 2015; 9: 995–1004. https://doi.org/10.1038/labinvest.2015.71

[15] XU F, LI J, NI M, CHENG J, ZHAO H et al. FBW7 suppresses ovarian cancer development by targeting the N(6)-methyladenosine binding protein YTHDF2. Mol Cancer 2021; 1: 45. https://doi.org/10.1186/s12943-021-01340-8

[16] CHEN X, LI XY, LONG M, WANG X, GAO ZW et al. The FBXW7 tumor suppressor inhibits breast cancer proliferation and promotes apoptosis by targeting MTDH for degradation. Neoplasma 2018; 2: 201–209. https://doi.org/10.4149/neo_2018_170228N149

[17] WU XZ, WANG KP, SONG HJ, XIA JH, JIANG Y et al. MiR-27a-3p promotes esophageal cancer cell proliferation via F-box and WD repeat domain-containing 7 (FBXW7) suppression. Int J Clin Exp Med 2015; 9: 15556–15562. https://www.ncbi.nlm.nih.gov/pubmed/26629048

[18] GOLDMAN M, CRAFT B, HASTIE M, REPEČKA K, MCDADE F et al. The UCSC Xena platform for public and private cancer genomics data visualization and interpretation. BioRxiv 2019. https://doi.org/10.1101/326470

[19] ZHANG L, ZHOU Y, CHENG C, CUI H, CHENG L et al. Genomic analyses reveal mutational signatures and frequently altered genes in esophageal squamous cell carcinoma. Am J Hum Genet 2015; 4: 597–611. https://doi.org/10.1016/j.ajhg.2015.02.017

[20] SONG B, CUI H, LI Y, CHENG C, YANG B et al. Mutually exclusive mutations in NOTCH1 and PIK3CA associated with clinical prognosis and chemotherapy responses of esophageal squamous cell carcinoma in China. Oncotarget 2016; 3: 3599–3613. https://doi.org/10.18632/oncotarget.6120

[21] YEH CH, BELLON M, NICOT C. FBXW7: a critical tumor suppressor of human cancers. Mol Cancer 2018; 1: 115. https://doi.org/10.1186/s12943-018-0857-2

[22] AGRAWAL N, JIAO Y, BETTEGOWDA C, HUTFLESS SM, WANG Y et al. Comparative genomic analysis of esophageal adenocarcinoma and squamous cell carcinoma. Cancer Discov 2012; 10: 899–905. https://doi.org/10.1158/2159-8290.CD-12-0189

[23] YOKOBORI T, MIMORI K, IWATSUKI M, ISHII H, TANAKA F et al. Copy number loss of FBXW7 is related to gene expression and poor prognosis in esophageal squamous cell carcinoma. Int J Oncol 2012; 1: 253–259. https://doi.org/10.3892/ijo.2012.1436

[24] YU H, LING T, SHI R, SHU Q, LI Y et al. [Expression of FBXW7 in esophageal squamous cell carcinoma and its clinical significance]. Zhonghua Zhong Liu Za Zhi 2015; 5: 347–351. https://www.ncbi.nlm.nih.gov/pubmed/26463024

[25] SINGH S, KUMAR S, SRIVASTAVA RK, NANDI A, THACKER G et al. Loss of ELF5-FBXW7 stabilizes IFNGR1 to promote the growth and metastasis of triple-negative breast cancer through interferon-gamma signalling. Nat Cell Biol 2020; 5: 591–602. https://doi.org/10.1038/s41556-020-0495-y

[26] AN HJ, LEE CJ, LEE GE, CHOI Y, JEUNG D et al. FBXW7-mediated ERK3 degradation regulates the proliferation of lung cancer cells. Exp Mol Med 2022; 1: 35–46. https://doi.org/10.1038/s12276-021-00721-9

[27] WANG Q, XU B, DU J, XU X, SHANG C et al. MicroRNA-139-5p/Flt1/Wnt/beta-catenin regulatory crosstalk modulates the progression of glioma. Int J Mol Med 2018; 4: 2139–2149. https://doi.org/10.3892/ijmm.2018.3439

[28] GIATROMANOLAKI A, KOUKOURAKIS MI, TURLEY H, SIVRIDIS E, HARRIS AL et al. Phosphorylated KDR expression in endometrial cancer cells relates to HIF1alpha/VEGF pathway and unfavourable prognosis. Mod Pathol 2006; 5: 701–707. https://doi.org/10.1038/modpathol.3800579

[29] AINIWAER Z, MAISAIDI R, LIU J, HAN L, HUSAIYIN S et al. Genetic polymorphisms of PGF and TNFAIP2 genes related to cervical cancer risk among Uygur females from China. BMC Med Genet 2020; 1: 212. https://doi.org/10.1186/s12881-020-01144-5

[30] CHEN S, YU M, JU L, WANG G, QIAN K et al. The immune-related biomarker TEK inhibits the development of clear cell renal cell carcinoma (ccRCC) by regulating AKT phosphorylation. Cancer Cell Int 2021; 1: 119. https://doi.org/10.1186/s12935-021-01830-1

[31] YAO S, DONG SS, DING JM, RONG Y, ZHANG YJ et al. Sex-specific SNP-SNP interaction analyses within topologically associated domains reveals ANGPT1 as a novel tumor suppressor gene for lung cancer. Genes Chromosomes Cancer 2020; 59: 13–22. https://doi.org/10.1002/gcc.22793

[32] LIN H, LIU S, GAO W, LIU H. DDIT3 modulates cancer stemness in gastric cancer by directly regulating CEBPbeta. J Pharm Pharmacol 2020; 6: 807–815. https://doi.org/10.1111/jphp.13243

[33] SONG Y, LIU S, ZHAO Z, ZHANG Y, YANG Y et al. Relationship between PPP1R15A gene polymorphism (rs611251) and Epstein-Barr virus-associated tumors. Acta Virol 2017; 4: 445–452. https://doi.org/10.4149/av_2017_407

[34] WANG HC, CHAN LP, WU CC, CHANG SJ, MOI SH et al. Silencing DNA Polymerase beta Induces Aneuploidy as a Biomarker of Poor Prognosis in Oral Squamous Cell Cancer. Int J Mol Sci 2021; 22: 2402. https://doi.org/10.3390/ijms22052402

[35] WANG S, CHEN X, FU Y, ZHANG H, LIU W et al. Relationship of ERCC5 genetic polymorphisms with metastasis and recurrence of gastric cancer. Rev Assoc Med Bras (1992) 2021; 11: 1538–1543. https://doi.org/10.1590/1806-9282.20210209

[36] CUI T, SRIVASTAVA AK, HAN C, WU D, WANI N et al. DDB2 represses ovarian cancer cell dedifferentiation by suppressing ALDH1A1. Cell Death Dis 2018; 5: 561. https://doi.org/10.1038/s41419-018-0585-y

[37] PAPA L, MANFREDI G, GERMAIN D. SOD1, an unexpected novel target for cancer therapy. Genes Cancer 2014; 1–2: 15–21. https://doi.org/10.18632/genesandcancer.4

[38] JIN T, KIM HS, CHOI SK, HWANG EH, WOO J et al. microRNA-200c/141 upregulates SerpinB2 to promote breast cancer cell metastasis and reduce patient survival. Oncotarget 2017; 20: 32769–32782. https://doi.org/10.18632/oncotarget.15680

[39] CHEN X, YU Q, PAN H, LI P, WANG X et al. Overexpression of IGFBP5 Enhances Radiosensitivity Through PI3K-AKT Pathway in Prostate Cancer. Cancer Manag Res 2020; 12: 5409–5418. https://doi.org/10.2147/CMAR.S257701

[40] CAO J, LIU X, YANG Y, WEI B, LI Q et al. Decylubiquinone suppresses breast cancer growth and metastasis by inhibiting angiogenesis via the ROS/p53/ BAI1 signaling pathway. Angiogenesis 2020; 3: 325–338. https://doi.org/10.1007/s10456-020-09707-z

[41] ABAD E, GRAIFER D, LYAKHOVICH A. DNA damage response and resistance of cancer stem cells. Cancer Lett 2020; 474: 106–117. https://doi.org/10.1016/j.canlet.2020.01.008

[42] CALCINOTTO A, KOHLI J, ZAGATO E, PELLEGRINI L, DEMARIA M et al. Cellular Senescence: Aging, Cancer, and Injury. Physiol Rev 2019; 2: 1047–1078. https://doi.org/10.1152/physrev.00020.2018

[43] HU L, LV X, LI D, ZHANG W, RAN G et al. The anti-angiogenesis role of FBXW7 in diabetic retinopathy by facilitating the ubiquitination degradation of c-Myc to orchestrate the HDAC2. J Cell Mol Med 2021; 4: 2190–2202. https://doi.org/10.1111/jcmm.16204

[44] ANUJA K, CHOWDHURY AR, SAHA A, ROY S, RATH AK et al. Radiation-induced DNA damage response and resistance in colorectal cancer stem-like cells. Int J Radiat Biol 2019; 6: 667–679. https://doi.org/10.1080/09553002.2019.1580401

[45] WANG L, CHEN R, LI G, WANG Z, LIU J et al. FBW7 Mediates Senescence and Pulmonary Fibrosis through Telomere Uncapping. Cell Metab 2020; 5: 860–877 e9. https://doi.org/10.1016/j.cmet.2020.10.004

[46] XIAO G, LI Y, WANG M, LI X, QIN S et al. FBXW7 suppresses epithelial-mesenchymal transition and chemo-resistance of non-small-cell lung cancer cells by targeting snai1 for ubiquitin-dependent degradation. Cell Prolif 2018; 5: e12473. https://doi.org/10.1111/cpr.12473

[47] ZHU Z, XU J, LI L, YE W, XU G et al. Effect of gastric cancer stem cell on gastric cancer invasion, migration and angiogenesis. Int J Med Sci 2020; 13: 2040–2051. https://doi.org/10.7150/ijms.46774

[48] ZHOU W, WANG G, GUO S. Regulation of angiogenesis via Notch signaling in breast cancer and cancer stem cells. Biochim Biophys Acta 2013; 2: 304–320. https://doi.org/10.1016/j.bbcan.2013.10.003

[49] NOWICKI M, WIERZBOWSKA A, MAŁACHOWSKI R, ROBAK T, GRZYBOWSKA-IZYDORCZYK O et al. VEGF, ANGPT1, ANGPT2, and MMP-9 expression in the autologous hematopoietic stem cell transplantation and its impact on the time to engraftment. Ann Hematol 2017; 12: 2103–2112. https://doi.org/10.1007/s00277-017-3133-4

[50] LIU T, LI K, ZHANG Z, PENG J, YANG J et al. Tetrandrine Inhibits Cancer Stem Cell Characteristics and Epithelial to Mesenchymal Transition in Triple-Negative Breast Cancer via SOD1/ROS Signaling Pathway. Am J Chin Med 2023; 51: 425–444. https://doi.org/10.1142/s0192415x23500222

[51] HAO J, YANG H, CAO Y, ZHANG C, FAN Z et al. IGFBP5 enhances the dentinogenesis potential of dental pulp stem cells via JNK and ErK signalling pathways. J Oral Rehabil 2020; 12: 1557–1565. https://doi.org/10.1111/joor.13047

[52] HAN Y, SUN T, HAN Y, LIN L, LIU C et al. Human umbilical cord mesenchymal stem cells implantation accelerates cutaneous wound healing in diabetic rats via the Wnt signaling pathway. Eur J Med Res 2019; 1: 10. https://doi.org/10.1186/s40001-019-0366-9

[53] HAN C, ZHAO R, LIU X, SRIVASTAVA A, GONG L et al. DDB2 Suppresses Tumorigenicity by Limiting the Cancer Stem Cell Population in Ovarian Cancer. Mol Cancer Res 2014; 12: 784–794. https://doi.org/10.1158/1541-7786.Mcr-13-0638

https://doi.org/10.4149/neo_2023_230104N8

FBXW7 inhibits the progression of ESCC by directly inhibiting the stemness of tumor cells

Yanghui BI[1,#], Yanfang YANG[2,#], Yong ZHANG[3,#], Caixia CHENG[4], Peisen TANG[5], Heng XIAO[6], Fajia YUAN[7], Weiwei WU[2], Bin YANG[8,*]

Supplementary Information

Supplementary Table S1. Primers used in study.

Gene name		Primer sequences
FBXW7	Forward primer	GGAACAGCAACAGCAACTCA
	Reverse primer	ACTCTCCTGGTCCATCTCCT
MDR1	Forward primer	AGAGGGGATGGTCAGTGTTG
	Reverse primer	CAATGGCGATCCTCTGCTTC
GAPDH	Forward primer	CCAGAACATCATCCCTGCCT
	Reverse primer	CCTGCTTCACCACCTTCTTG
ANGPT1	Forward primer	GAAGGGAACCGAGCCTATTC
	Reverse primer	GGGCACATTTGCACATACAG
FLT1	Forward primer	ACAGTGGCCATCAGCAGTTC
	Reverse primer	GACGATGGTGACGTTGATGT
KDR	Forward primer	GTGACCAACATGGAGTCGTG
	Reverse primer	TGCTTCACAGAAGACCATGC
BMI1	Forward primer	CCAGGGCTTTTCAAAAATGA
	Reverse primer	CCGATCCAATCTGTTCTGGT
IGFBP5	Forward primer	GAGCTGAAGGCTGAAGCAGT
	Reverse primer	GAATCCTTTGCGGTCACAAT
SERPINB2	Forward primer	GTTCATGCAGCAGATCCAGA
	Reverse primer	CGCAGACTTCTCACCAAACA
SOD1	Forward primer	AGGGCATCATCAATTTCGAG
	Reverse primer	ACATTGCCCAAGTCTCCAAC
TBX2	Forward primer	TATCAGATCCCGGTCACCAT
	Reverse primer	CACCAGTCTCTGGATGCTCA
DDB2	Forward primer	TCAAGGACAAACCCACCTTC
	Reverse primer	GTGACCACCATTCGGCTACT
ERCC3	Forward primer	GCGGCAGAGATTCTTGGTAG
	Reverse primer	GGCCCCAGACATAGAACTCA
ERCC5	Forward primer	CAGACACAGCTCCGAATTGA
	Reverse primer	TTCTGGGTTTTTCGTTTTGC
POLB	Forward primer	GAGAAGAACGTGAGCCAAGC
	Reverse primer	CGTATCATCCTGCCGAATCT
PPP1R15A	Forward primer	GGTCCTGGGAGTATCGTTCA
	Reverse primer	CAGGGAGGACACTCAGCTTC
TEK	Forward primer	TTTGAAGCACCTGCCTTTCT
	Reverse primer	CAGGAACAGGTGACTGCTCA
DDIT3	Forward primer	GCTGCTGGAGACCCTCTATG
	Reverse primer	ATCCATCCAAACCAGCTGTC
PGF	Forward primer	CAAGACCCTCAAGCCAGAAG
	Reverse primer	CGGATCAACTCCACCTGTCT